Course Name: Basics of Crop Breeding and Plant Biotechnology

Professor Name: Dr. Joydeep Banerjee

Department Name: Agricultural and Food Engineering

Institute Name: Indian Institute of Technology Kharagpur

Week: 08

Lecture-36: Types of Vectors used in Plant Transformation and Selectable Marker Gene

Hello everybody. Welcome to SWAYAM NPTEL, an online course on the Basics of Crop Breeding and Plant Biotechnology i.e., Introduction to Genetic Engineering and Plant Tissue Culture. Here we will be discussing different types of marker genes, and the requirements of plant tissue culture, what are the basic requirements needed for plant tissue culture. So, the concepts that will be covered under this particular topic those are, first we will start our discussion with the Ti plasmid of *Agrobacterium tumefaciens*. In our last lecture, we discussed the Ti plasmid and today we will start our discussion from that, and then how can you modify it. So, that the plant molecular biologist can utilize those Ti plasmids will be discussed.

Then we will be discussing, the binary and cointegrate vectors which are different vectors, that are used for the *Agrobacterium*-mediated plant transformation process. Then, we will be discussing positive and negative selection markers, different marker genes that are available, which are utilized in plant tissue culture and what are the basic requirements of plant tissue culture will be discussed.

So, this is the Ti plasmid of *Agrobacterium* tumefaciens its size is huge. So, it's close to 200 kb, its total size, we know that the plasmid is a double-stranded circular DNA. Ok! It is available within the bacterial cell, and it is different from the genomic DNA of the bacteria. Ok! So, each plasmid must have an origin of replication. So, that it can replicate within the particular host cell. So, now let us see what are the things available in Ti plasmid. So, the major thing available in Ti plasmid is, the origin of replication,

and then the virulence region is available, which means different genes are there *virA*, *virD*, *virE*, and other different genes are there.

Some of them are involved in plant-bacteria interaction, some of them are involved in different phosphorylation and dephosphorylation processes, and some of them are involved in transferring the DNA from bacteria into the plant. Ok! So, different genes are available within the virulence region. In addition to that the important thing that we have discussed in our previous class, i.e., the region available between the left border and the right border region. So, basically, this region whatever is available between the left border and the right border region, is transferred from this particular gram-negative bacterium into the plant genome. And once this is transferred into the plant genome then as different genes of auxin and cytokine are available.

So, auxin production, and cytokine synthesis will be more, will be rapid within the plant cell and a rapid cell division is taken place uncontrolled cell division is taken place. And, finally, we can see the gall formation in the plants where the *Agrobacterium* has caused such type of infection. Now within this left border and right border region, the opine genes are there, are responsible for some opine synthesis. And if you see that the Ti plasmid of *Agrobacterium*, their opine catabolism genes, are also there means those genes can catabolize the opines. So, that the product could be utilized by the bacteria for its survivability. Ok!

So, these are the things available in Ti plasmid. Now, if you carefully see for successful transfer of the region available between the left border and the right border, this region is also known as the T-DNA region, the transfer DNA region. For a successful transfer, this virulence gene or virulence region is needed. Ok! And, scientists have found that if we put any other genes in spite of auxin, cytokine, and opines which are available over here in the T-DNA region. If we put, suppose any gene of interest under a particular promoter and a terminator, if we put it over here then that particular gene of interest could be delivered into the genome of the targeted plant through this *Agrobacterium*-mediated

gene transfer method.

Because this left border and right border region is very much crucial, whatever is available in between could be transferred. So, scientists have modified the *Agrobacterium tumefaciens*, Ti plasmid they have done the modification to reduce the size and to remove the unwanted regions. Ok! So, somehow, they can generate some vector through which they can deliver different target genes into the targeted plant species. So, now we will see about two different types of modifications, that were done using this basic concept of *Agrobacterium tumefaciens* Ti plasmid.

So, these are the types of vectors used for plant transformation, first, we will be discussing about binary vectors. So, those are highly popular, binary means from the name itself it signifies that two-vector system is available over there, two vectors are used over there. In one vector, basically in the basic binary vector will be having the, origin of replication, and two origins of replications are there. First of all, if we have to do, if you have to utilize this binary vector for plant transformation, initially we have to design a construct having our gene of interest with its promoter with its terminator those things. So, those genes of interest could be placed within the MCS region, the multiple cloning sites region. In multiple cloning sites region, different restriction endonuclease sites are there.

So, using those restriction endonuclease sites, gradually we can put the promoter the gene of interest, and the terminator. And, the PSM gene, the plant-selectable marker gene, should be also available there. Earlier, we have discussed about, the selectable marker gene which could be placed within the plasmid, so that we can do the selection of the transformed bacteria. Here the plant-selectable marker genes are used so that with the help of those genes, we can trace those putative transformants where our transgene or where our gene of interest is being delivered. So, the plant-selectable marker genes are used for the selection process, later on, we will be discussing what are the different types of selectable marker genes used in plant systems. Ok!

So, if you see carefully in this binary vector here left border and right border regions is available. So, whatever will be placing in between that will be transferred into the plant genome, but if you just recall the *Agrobacterium* Ti plasmid their virulence genes were also available, but in this basic binary vector the virulence genes are not available. So, for a successful gene transfer method, we need a helper Ti plasmid. In this helper Ti plasmid, different vir genes are available along with the origin of replication also. So, using this type of system easily we can transfer our target gene through *Agrobacterium* into the desired plants.

Maybe we can transfer it in the rice genome, we can transfer it in cotton, in brassica, in tobacco in different genomes we can deliver our target genes. Ok! So, if you think about the binary vector some successful binary vectors are available, like different vectors under pCAMBIA series, pCAMBIA 1300, pCAMBIA 1301, then pCAMBIA1391Z, these are different binary vectors that are commonly used. The 1300 and 1301 are used for delivering specific gene of interest, while 1391Z this one is used for promoter characterization later on we will be discussing this part. Now, this is the cointegrate type of vector. Ok! Here basically within this cointegrate plasmid, both the things are there, the plant-selectable marker gene and the gene of interest both of them are available the arrow indicates the promoter region.

This promoter will express the plant-selectable marker gene while this promoter will express the gene of interest. Definitely, thereafter terminator will be also there this is the terminator for the plant selectable marker gene and this one is the terminator, for the gene of interest. It is available within the left border and right border region and then bacterial selectable marker gene must be there, also and several vir genes are available over there means within the bacterial cell by recombination process, this cointegrate vectors are formed. Their origin of replication is also needed so that it can replicate within the bacterial system. So, the cointegrate vector system involves the, integration of a T-DNA region into the plant genome.

The T-DNA is a segment of DNA that carries the gene of interest and is transferred from

the *agrobacterium* vector to the plant cells during the infection process. So, now gradually we will start our discussion on different selectable marker genes. First, once the basic concept of selectable marker genes came, the selectable marker gene are usually an integral part of plant transformation because once we will try to do any type of plant transformation. Let us assume we are trying to deliver some genes through *agro bacterium* into the tobacco genome. In tobacco transformation, is done by leaf disc method, the leaf is cut into small pieces, one-centimeter square or a little bit smaller than that in this way the leaves are chopped and then the leaves are infected with the *Agrobacterium* solution harboring our particular plasmid harboring our transgene. Ok!

So, thereafter gradually the *Agrobacterium* cell will start infection in the presence of acetosyringone and finally, the plantlets will be coming from different cut surfaces of the tobacco leaves. And, if you try to do it within a month you will see hundreds of plantlets are coming, from different parts of the tobacco leaves, of the cut tobacco leaves. Ok! So, out of those hundreds of plantlets, maybe not all means, not all the plants might be transformants, means within all the plants our transgene may not be delivered. Ok! So, some selection process is needed maybe in 10 % plant our transgene has been delivered. So, we need to identify those plants, otherwise, if we generate 1000 tobacco plants thereafter further screening will be too much costly, and too much difficult out of those 1000s in which specific 100 plants, our transgene has been delivered, this selection process will be cumbersome.

So, the selectable marker gene is used, so that we can screen them from the beginning. Ok! So, they are present in the vector along with the target gene, earlier if you see the binary vector or cointegrate vector, it will be clear that within the left border and right border region along with the target gene, the selectable marker genes, are also there. In the majority of cases, the selection is the basis of survival of the transformed cells, when grown on a medium containing the toxic substance. So, this concept initially came, once the selectable marker gene-related study was initiated means some toxic substance was given on the media or is given in the media where most of the plant cells, where our transgene will not be delivered would be killed, while those plant cells where our

transgene has been delivered they will produce some they will produce the product of that particular selectable marker gene, which can degrade the toxic chemicals or which can degrade the proper antibiotics so that those cells could be survived. So, this is due to the fact that a selectable marker gene confers resistance to toxicity in the transformed cells, while the non-transformed cells get killed as I have mentioned just earlier.

A large number of selectable marker genes are available, and they are grouped into three categories. So, these selectable marker genes, whatever we are discussing so far those are known as negative selectable marker, those are known as negative selectable marker genes because they have some negative impact on the untransformed cells, while the transformed cells where our transgene has been delivered, it can sustain that toxic condition. Ok! So, under this category, the antibiotic resistance genes are there the antimetabolite marker genes, are there herbicide resistance genes are there, and so on. So, let us start our discussion on some plant-negative selectable marker genes. Mostly the initial sets of experiments or initial selectable marker genes that were used were antibiotic-selectable markers because antibiotics can effectively inhibit the protein synthesis of cellular organelles.

So, among them neomycin phosphotransferase II gene or the *nptII* gene, it has been very popular, even now also this specific antibiotic resistance gene is used as a selectable marker gene for plant transformation. The most widely used selectable marker gene, including the enzyme neomycin phosphotransferase II, it confers resistance to antibiotic kanamycin and on the media we can put G418 also then the transfer or we have to use kanamycin selection for plants. The transformants and the plants derived from them can be checked by applying kanamycin solution or G418 solution and the resistance progeny can be selected we can use G418 also and the resistance progeny can be selected. So, which will be putative transformants the resistant ones will be putative transformants while the non-transformants are mostly killed if we add kanamycin solution in the media. Then another important gene i.e., *hpt* gene hygromycin phosphotransferase gene, it is more toxic than neomycin and therefore, can kill non-transformed plant cells much faster.

Ok!

In the case of *Arabidopsis*, for different transformations in Arabidopsis, *nptII* genes are mostly used while in the case of rice transformation, mostly hygromycin phosphotransferase are used, but here toxicity is more. So, recently a lot of scientific communities have shifted from using *hpt* gene to using *npt* gene. So, this *npt* gene or neomycin phosphotransferase is being more famous compared to *hpt* gene, but in case of *hpt* gene the selection is more robust. Ok! Then, third one is aminoglycoside adenyltransferase or *aadA* gene. So, it confers resistance to transform plant cells against the antibiotic's streptomycin and spectinomycin means, the transformed plant cells will sustain both this particular antibiotic streptomycin and spectinomycin.

So, these are some plant-negative selectable marker genes. Now let us discuss a little bit about some plant-positive selectable marker genes. Among them one gene is named as *man-A*, it produces a phosphomannose isomerase enzyme, the *man-A* gene it produces a phosphomannose isomerase enzyme. So, the *man-A* gene codes for the enzyme it is also known as phosphomannose isomerase i.e., PMI. So, PMI could be used as a positive selectable marker gene, a positive selectable marker gene what is that I will tell you just now.

So, in the presence of mannose, a PMI particular enzyme, it converts mannose 6-phosphate into fructose 6-phosphate which is immediately incorporated into the plant metabolic pathway metabolic pathway as the carbohydrate source. Now how positive selectable marker gene work? Suppose, this is our plate here different putative transformants are there, suppose out of them, only within this one our transgene has been delivered. So, in a negative selectable marker gene, what will happen? In negative selectable marker, we will add that particular antibiotic or herbicide within the media and the non-transformants will be killed, while the transformants will survive its growth will be retarded, but they will survive and it can sustain. While, in the case of a positive selectable marker gene, the non-transformants will not be killed easily, but some specific gene or some specific protein or enzymes will be produced within the positive one. And

with the help of that enzyme or protein the positive one can grow faster they can utilize the media easily and they can grow faster while the rest of them will show stunted growth or they cannot grow properly.

So, this is the way, in which positive selectable marker gene and negative selectable marker gene works. So, over here if mannose is given in the media, then in those plants where the PMI gene, has been used or the *man-A* gene has been used it can convert mannose 6 phosphate into fructose 6 phosphate. And, fructose-6-phosphate is a part of different metabolic pathways that could be easily utilized by the plant. While, in those plants, which are non-transformants where our transgene has not been delivered, they cannot utilize mannose-6-phosphate or they cannot utilize mannose.

So, their growth will be retarded. Ok! So, now another example of a positive selectable marker gene is the xylose isomerase or *xyl*A gene. It catalyzes the isomerization of D-xylose to D-xylulose, in the pentose phosphate pathway. It enables the utilization of xylose as a carbon source. So, in an earlier case, mannose could be used as a carbon source over here xylose could be used as a carbon source. If we use the *xylA* gene, or xylose isomerase gene as a positive selectable marker.

Then, another one is the *DOG1* gene. So, 2- deoxyglucose 6 phosphate phosphatase, it is also used as a positive selectable marker and it has been used in different plants like tobacco, and potatoes in their transformation. In transgenic plants, the gene gives resistance against 2-deoxyglucose or 2-DOG, when this particular positive selectable marker genes are over-expressed. So, now coming to another type of negative selectable marker, it is also a negative selectable marker i.e., antimetabolite marker gene. Over here dihydrofolate reductase, *dhfr* gene could be used, the enzyme dihydrofolate reductase produced by this particular gene is inhibited by the anti-metabolite methotrexate. This *dhfr* gene fused with CaMV35S promoter, in construct for each and every gene, either for your gene of interest or for the selectable marker gene, a promoter should be there.

So, in a particular study, it has been found that once the *dhfr* gene was expressed on the

CaMV35S promoter, it resulted in methotrexate, methotrexate-resistant marker, which means once the methotrexate was applied in the media, those plants having this particular gene they were surviving, means it was resistant to methotrexate which can be used for selection of the transformed plants. While, for the rest of the plants, where this *dhfr* gene will not be incorporated, those will be eventually killed. Then coming to herbicide resistance markers, which are also negative selectable markers. So, genes that confer resistance to herbicides are used as a marker for the selection of transgenic plants. Some examples are mentioned here, first one is phosphinothricin acetyltransferase or pat gene i.e., commonly known as *bar* gene.

So, it produces an enzyme, phosphinothricin acetyltransferase. So, some common herbicides, that are used in different field experiments, are bialaphos, phosphinothricin, and glufosinate, which are commonly used herbicides. So, the pat/bar gene i.e., phosphinothricin acetyltransferase enzyme-producing genes, they convert these herbicides into an acetylated form that are non-herbicidal, which means that does not kill the green plants. Ok! Its herbicidal activity is reduced, if the pat gene is there, due to acetyl transfers, the acetyl group is transferred. So, the pat/bar gene confers resistance to the transformed plant cells, if, in the media, we can use this type of herbicides like glufosinate or phosphinothricin, bialaphos those things.

The next one is Bromoxynil nitrilase, the name of this gene is the *bxn* gene. So, the herbicide Bromoxynil inhibits photosynthesis in PS-II, and is basically, inhibited by this particular herbicide Bromoxynil. So, the Bromoxynil nitrilase means this particular enzyme produced by this herbicide-resistant gene, inactivates the Bromoxynil herbicide, and the gene *bxn*, can be successfully used as a selectable marker for the selection of transformed plants also.